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The *N*-Terminal Amino Acid Residues of Gelatin

2. THERMAL DEGRADATION

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Gelatin in solution undergoes changes in the presence of proteinases or at elevated temperatures, especially at acid or alkaline pH values, to which the general term 'degradation' is given. The effect of degradation in reducing the solution viscosity, and in impairing the gel-forming power, is so readily demonstrated for gelatin, as compared to other proteins, that it has received considerable study. The change in physical properties has frequently been used as a measure of the extent of the degradation reaction (Sheppard & Houck, 1930, 1932; Pouradier & Venet, 1952 and previous papers; Gerngross & Brecht, 1922; Ames, 1947). In view of the uncertain relationships between such properties and the rate of bond breaking the results obtained must be looked on as only empirical, although of considerable practical value.

The degradation of gelatin gives rise to an increase of α-amino groups and this property has been used for assessing the degree of hydrolysis. Northrop (1921*a*, *b*) has used the formol-titration method. Greenberg & Burk (1927) have employed the Van Slyke nitrous acid method. The predominance in gelatin of ε-amino groups of lysine and hydroxylysine residues would make small changes in α-amino groups difficult to detect by these techniques (the proportion of α- to ε-amino groups in material of molecular weight 50000 is 1:20). In addition, neither method will distinguish between α-amino groups formed by rupture of peptide bonds and any other amino groups such as would be involved in the possible conversion of an arginine residue into ornithine. These methods have been mainly of value in studying extensive degradation where the number of α-amino groups released has become large.

The *N*-terminal residue method as described in the preceding paper (Courts, 1954) is likely to be of value in following the course of protein degradation, especially in the early stages where preferential breaking of labile bonds may occur. Thus the identification of the *N*α-2:4-dinitrophenylamino acids would give some information as to the nature of the weaker peptide bonds and also a measure of the extent of hydrolysis. This paper reports some experiments on the controlled thermal degradation of gelatin using the *N*-terminal residue method. The theoretical considerations arising from the results are discussed and related to the physical properties of the gelatins obtained.

MATERIALS AND METHODS

Gelatin. A de-ionized ossein gelatin of isoelectric point pH 5·1 was used. This was prepared from bone collagen and characterized by its Bloom jelly strength (6·67% concentration), 217 g., and viscosity (40° and 6·67% concentration) 7·05 centipoise.

Bone collagen. This was prepared by the method of Eastoe & Eastoe (1954). I am indebted to Dr J. E. Eastoe for a gift of this material.

Technique of degradation. The extent of protein hydrolysis is determined by time and temperature of heating and by pH of solution. Each of the three was varied in turn while the other two were kept constant. Degradations were carried out in sealed tubes using 5% (w/v) solutions.

The pH of gelatin solutions shifts slightly during degradation (Ames, 1947) towards the isoelectric pH, the shift becoming very large at high pH values. This effect is minimized when sealed tubes are used, but no attempt was made to maintain the pH values constant during degradation with buffers since this was likely to introduce unwanted complications. All pH measurements were made with a Marconi pH meter (Marconi Instruments, London) using

a Doran 'Alkacid' glass electrode (L.S.B. Components Ltd., Stroud, Glos.) and a saturated calomel electrode (Cambridge Instrument Co. Ltd., London). When heating is effected at the isoelectric point, no change in pH occurs, at least up to 100° during 5 days. Consequently, when gelatin solutions were to be degraded at constant pH, the isoelectric pH was chosen.

Wherever possible, boiling solvents were used to attain constant temperatures, viz., $CHCl_3$ (60°), benzene (80°), water (100°). Temperatures of 75 and 90° were maintained by a thermostatically controlled oven to within ±0·25°.

N-Terminal residue determinations. These were carried out essentially as described by Courts (1954). In the reaction between partially hydrolysed gelatin and 1:2:4-fluorodinitrobenzene the precipitation of dinitrophenylgelatin (DNP-gelatin) was not successfully attained simply by acidification, and magnesium sulphate (saturated at and added at 40°, 150 ml.) was employed. To avoid overloading the column during chromatographic separation of DNP-amino acids, the amount of DNP-gelatin taken for hydrolysis was decreased to 100–300 mg. for the more degraded materials.

Rigidity determinations. These were done at 10°, after 18 hr. maturing, by the method of Saunders & Ward (1954). Mr P. R. Saunders kindly undertook the measurements on the samples, values of which are given in Tables 2, 4 and 5.

Reduced viscosity. The reduced viscosity η_i is expressed as $1/c \ln(\eta_{rel.})$, where c is concentration (%, w/v) and $\eta_{rel.}$ is $\eta_{solution}/\eta_{solvent}$. The conditions chosen were c approx. 0·5 in M-NaCl at pH 9. In these conditions the reduced viscosity is almost independent of c, and does not change with small variations of pH and salt concentration. The measurements were made at 35° in a no. 1 Ostwald viscometer (British Standards Institution (1937) 188) and these were kindly undertaken by Dr G. Stainsby. Variations in reduced viscosity may be regarded as closely corresponding to variations in M_w, the weight-average molecular weight (cf. Pouradier & Venet, 1952).

RESULTS

Degradation as a function of pH. (pH range, 1–12; temperature, 75°; time, 24 hr.) The conditions of time and temperature were chosen to give a substantial change of end-group values without degrading the protein to small peptides. Table 1 shows the extent of pH shift during 24 hr. heating. A comparison of the degradation results is shown in Table 2 as residues/100 000 g. gelatin, and again in Table 3 indicating the *N*-terminal residues as a percentage of the number of residues which could theoretically occur as end groups. Certain physical properties of the degraded protein are shown in

Table 1. pH *shift of gelatin solutions during degradation*

pH initial	1·1	2·1	3·1	4·1	5·1	7·0	9·1	10·2	11·2	12·2
pH final	1·4	2·8	3·4	4·3	5·1	6·7	8·7	9·6	9·9	10·4

Table 2. *Liberation of* N*-terminal groups and physical behaviour on degradation of gelatin as a function of* pH

For conditions, see text. End groups are expressed as moles/100 000 g. gelatin. M_n is number-average molecular weight calculated from end groups. η_i is reduced viscosity.

pH	1	2	3	4	5	7	9	10	11	12	Undegraded
Glycine	18·54	11·68	10·28	2·90	1·30	1·25	1·45	2·48	7·76	14·07	0·95
Serine	2·41	0·67	0·31	0·33	0·22	0·21	0·20	0·31	1·11	2·03	0·29
Threonine	2·53	0·58	0·36	0·21	0·16	0·14	0·13	0·21	0·42	1·26	0·12
Alanine	2·11	2·63	0·16	0·98	0·17	0·07	0·21	0·25	0·43	1·82	0·14
Aspartic acid	4·14	4·41	0·88	0·46	0·22	0·19	0·13	0·13	0·23	0·52	0·08
Glutamic acid	1·23	0·54	0·50	0·27	0·13	0·09	0·12	0·17	0·46	0·52	0·14
Others	0·82	0·45	1·10	0·23	0·05	0·05	0·00	0·05	0·22	0·41	0·00
Total	31·78	20·98	13·59	5·38	2·25	2·00	2·24	3·61	10·63	20·63	1·73
M_n	3000	5000	7000	19 000	45 000	50 000	45 000	28 000	9000	5000	58 000
η_i (½%)	0·031	0·077	0·090	0·143	0·33	0·35	0·30	0·20	0·114	0·042	0·57
M_n/initial M_n (%)	5·5	8·2	13	32	77	87	77	48	16	8·4	—
η_i/initial η_i (%)	5·4	13·5	16	25	58	61	53	35	20	7·4	—
Rigidity × 10^{-3} (dynes/cm.²)	Did not set			1·7	—	31·4	16·7		Did not set		75·4

Table 3. N*-Terminal residues as % of total residues of each kind present in gelatin degraded at different* pH *values (Table 2)*

pH	1	2	3	10	11	12	Undegraded
Glycine	5·0	2·9	2·7	0·65	2·1	3·7	0·25
Serine	7·5	2·1	0·95	1·3	3·4	6·3	0·70
Threonine	11·8	2·9	1·8	1·1	2·1	6·3	0·56
Alanine	2·1	2·6	0·16	0·25	0·43	1·8	0·14
Aspartic acid	9·0	9·6	1·9	0·26	0·49	1·2	0·17
Glutamic acid	1·8	0·90	0·72	0·25	0·65	0·81	0·20

Table 4. *Degradation of gelatin as a function of temperature*

For conditions see text. End groups are expressed as moles/100000 g. gelatin. M_n is number-average molecular weight calc. from end groups. η_i is reduced viscosity.

N-terminal residues	60°	80°	90°	100°
Glycine	1·39	2·64	3·64	6·06
Serine	0·24	0·27	0·37	0·47
Threonine	0·12	0·20	0·32	0·33
Alanine	0·17	0·53	1·26	2·17
Aspartic acid	0·16	0·54	1·00	1·89
Glutamic acid	0·12	0·16	0·33	0·36
Others	0·00	0·08	0·38	0·54
Total	2·20	4·42	7·30	11·82
Mol.wt. (M_n)	45 500	22 600	13 700	8500
Reduced viscosity	0·36	0·19	0·14	0·10
M_n/initial M_n (%)	79	39	24	15
η_i/initial η_i (%)	64	35	25	18
Rigidity $\times 10^{-3}$ (dynes/cm.^{2})	46	1·5	Did not set	

Table 5. *Degradation of gelatin as a function of time*

For conditions see text. End groups are expressed as g. residues/100000 g. gelatin. M_n is number-average molecular weight calculated from end groups. η_i is reduced viscosity.

	pH 5·1						pH 4·3					
	16 hr.	1 day	2 days	3 days	4 days	5 days	16 hr.	1 day	2 days	3 days	4 days	5 days
Glycine	2·23	2·47	3·64	5·00	6·14	6·58	3·43	4·25	5·32	7·32	8·89	10·84
Serine	0·28	0·39	0·37	0·43	0·45	0·63	0·49	0·58	0·65	0·75	0·87	0·91
Threonine	0·16	0·24	0·32	0·36	0·42	0·43	0·24	0·19	0·23	0·23	0·46	0·73
Alanine	0·83	0·79	1·26	1·80	2·05	2·18	1·27	1·93	2·53	3·14	3·49	4·03
Aspartic acid	0·49	0·65	1·00	1·61	2·50	2·45	0·81	1·11	1·71	3·03	4·87	7·60
Glutamic acid	0·19	0·17	0·33	0·26	0·23	0·60	0·27	0·36	0·52	0·54	0·86	1·24
Others	0·21	0·18	0·38	0·40	0·33	0·95	0·46	0·48	0·62	0·78	0·50	0·92
Total	4·39	4·89	7·30	9·86	12·12	13·82	6·99	8·90	11·58	15·79	19·94	26·27
M_n	22 700	20 400	13 700	10 100	8300	7200	14 300	11 200	8600	6300	5000	3800
η_i	0·20	0·175	0·14	0·119	0·10	0·094	0·13	0·11	0·096	0·088	0·081	0·073
M_n/initial M_n (%)	40	35	24	17	14	12·5	24	19	14·5	11	8·5	6·5
η_i/initial η_i (%)	36	31	25	21	18	17	24	20·5	17·1	15·7	14·5	13·0
Rigidity $\times 10^{-3}$ (dynes/cm.^{2})	7·9	2·7	Did not set				Did not set					

Table 2 and may be related to the corresponding results obtained by the *N*-terminal residue method.

Degradation as a function of temperature. (Temperatures 60, 80, 90, 100°; time, 48 hr.; pH 5·1.) Results are shown in Table 4.

Degradation as a function of time. (Times 16 hr., 1, 2, 3, 4, 5 days; temperature, 90°; pH, (*a*) 5·1, (*b*) 4·1 (initial)—in this instance the pH rose to 4·3 during 16 hr. and remained constant during 5 days.) Results at both pH values are shown in Table 5.

Calculation of M_n. A gelatin sample showing a total of T moles of *N*-terminal residues/100000 g. will show 1 mole in $100000/T$ g. If it is assumed that one molecule carries one *N*-terminal residue, then it follows that $100000/T$ represents the molecular weight of the sample. It is unlikely that this sample will be homogeneous with respect to molecular distribution. Consequently $100000/T$ represents a number-average molecular weight value.

DISCUSSION

Although small changes in temperature and time of heating may have a marked effect on the rate of release of free α-amino groups, changes in rate are more readily obtained by varying the pH of solution. Fig. 1 shows a smooth relationship to exist between peptide-bond breaking and pH. The rate is minimal at pH 7. Beyond the range pH 5–9, degradation increases sharply so that at pH 4, the number-average molecular weight, M_n, is only one-third of its original value (assuming one α-amino group/molecule) while at pH 3 it is one-eighth.

Glycine is the most frequently found *N*-terminal residue, accounting for over half of the total. The extent of breakdown of peptide bonds involving the amino groups of the other amino acids depends on whether acid or alkaline conditions of hydrolysis are used. The sum total of amino groups of aspartic and

glutamic acid residues shows a slight predominance over that for serine and threonine residues when degradation occurs in the acid range. Above pH 7, serine and threonine become much more important than the dicarboxylic acids (Fig. 2).

The preparation of oxhide collagen by the method of Bowes & Kenten (1948) and of ox-bone collagen by the method of Eastoe & Eastoe (1954) may lead to slight degradation of the material. Examination of the end groups of these materials (Courts, unpublished) showed glycine as the main *N*-terminal residue together with small amounts of serine, threonine, aspartic acid and glutamic acid. There is no indication, however, as to the order of release of these residues at this stage. Further hydrolysis of the collagens adds alanine to the list of *N*-terminal residues giving a qualitative correspondence with those present in gelatin. When gelatin is degraded, the next *N*-terminal residues to appear after the six given above are valine and phenylalanine respectively. Partridge & Davis (1950) have observed in studies of the acid hydrolysis of certain

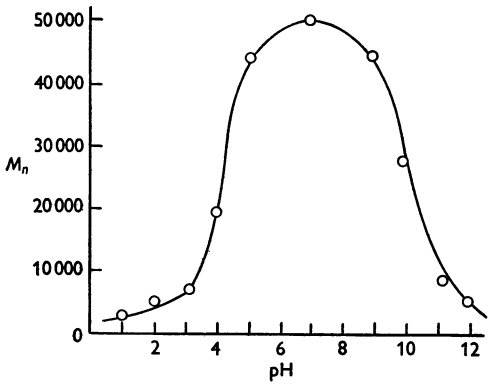

Fig. 1. Number-average mol.wt. of gelatin (M_n) after heating in solution for 24 hr. at 75° at different pH values. (M_n initially 58000.)

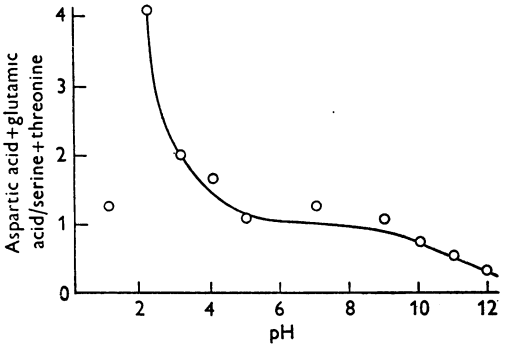

Fig. 2. The ratio of dicarboxylic amino acids to hydroxyamino acids released as *N*-terminal residues after heating gelatin at different pH values for 24 hr. at 75°.

proteins (including gelatin) that aspartic acid is preferentially released in the free state and that this is often followed by preferential release of glutamic acid. An extension of this work with gelatin in these laboratories has shown that free amino acids are released in the order aspartic acid, glutamic acid, glycine, serine and alanine (serine was possibly contaminated with threonine). These are the same residues which occur as the principal *N*-terminal groups. It is reasonable to assume, therefore, that these residues are released as end groups as an intermediate stage before the complete separation as free amino acids. It is also very likely that the order in which they are detected in the free amino acid state is the same as the order (of descending magnitude) in which they are determined as *N*-terminal groups.

The lability of certain peptide bonds has been studied by Levene, Synge and others using dipeptide hydrolysis. This and related work has been recently reviewed by Sanger (1952). Peptides so far examined involving glycine and alanine residues are relatively unstable irrespective of whether these are linked through —CO— or —NH— groups; glycylglycine is particularly unstable. Valyl peptides, on the other hand, appear to have a marked stability. Peptide bonds involving proline residues were found to exhibit unusual stability by Consden, Gordon, Martin & Synge (1947). Certain characteristics of gelatin degradation are in accord with this evidence. The ease with which gelatin is hydrolysed, for example, might be due in some measure to the large proportion of glycine residues (26·9 %) and the small proportions of valine and (leucine + isoleucine) residues (3·3 and 5·2 %). The predominance of glycine as an *N*-terminal residue would support this view. In a protein containing such a large proportion of residues of proline (14·8 %) and hydroxyproline (14·5 %), it might be expected that these amino acids would appear as end groups. Their non-appearance as *N*-terminal residues supports the view of Consden *et al.* (1947) of the great stability of peptide bonds involving proline. It is interesting that of the several peptides which have been isolated from gelatin partial hydrolysates (Synge, 1943; Gordon, Martin & Synge, 1943; Heyns, Anders & Becker, 1951; Schroeder, Honnen & Green, 1953) only one peptide is definitely identified as carrying hydroxyproline as the *N*-terminal residue while few appear to terminate with a proline residue. Smith (1953) has drawn attention to the ease with which many compounds of proline and hydroxyproline cyclize to form diketopiperazines. It is unlikely that similar cyclization occurs in polypeptides or proteins, although the possibility cannot be excluded. In the same way, Le Quesne & Young (1950) have shown that aqueous solutions of α-glutamyl peptides readily cyclize, probably into

stable pyrrolidone derivatives, but no such formation has been reported for α-glutamyl polypeptides.

The rate of breakdown of peptide bonds with temperature is obtained from Table 4. The temperature dependence of the rate of hydrolysis (k) is expressed by Arrhenius's equation

$$\frac{d \ln k}{dT} = \frac{A}{RT^2}$$

where A is the activation energy, R the gas constant and T the absolute-temperature. Under the present circumstances, the rate of hydrolysis may be replaced by the change in the number of end groups in a given time (r) measured in any consistent units, so that

$$\frac{d \ln r}{dT} = \frac{A}{RT^2}.$$

On integration this becomes

$$\ln r = -\frac{A}{RT} + \text{constant},$$

A, therefore, may be calculated from the slope of a graph of $\log_{10} r$ against the reciprocal of T. The plot of $\log_{10} r$ against $1/T$ is shown in Fig. 3 and the slope is given by $A/4{\cdot}61$. The value obtained for the activation energy of gelatin hydrolysis, 18 960 cal./mole is in fair agreement with certain previous results (Table 6).

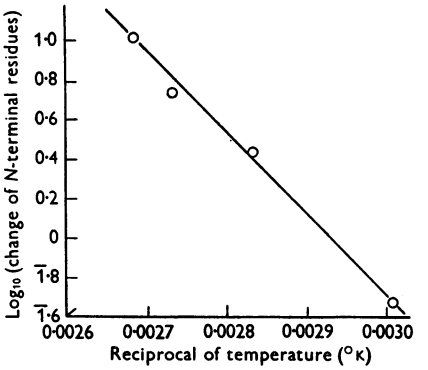

Fig. 3. The relationship between $\log_{10}$ (change of N-terminal residues) and the reciprocal of absolute temperature, the slope of which gives the activation energy of gelatin hydrolysis at pH 5·1.

Table 6. *Activation energies for hydrolysis of peptide bonds in gelatin (involving* —NH— *groups of specified residues)*

	Present work	Sheppard & Houck (1932)	Calc. from Greenberg & Burk (1927)
All bonds	18 960	19 920* – 26 540	2 480†
Glycine	15 360	—	—
Aspartic acid	22 670	—	—
Alanine	26 320	—	—
Serine and threonine	13 400	—	—

* By viscosity. † By amino nitrogen.

A plot of the change of total N-terminal residues with time (Fig. 4) shows an initial rapid increase which may be assumed to be due to the most labile peptide bonds. Once this stage of breakdown has been reached, hydrolysis proceeds at a steady rate. A somewhat similar general picture is reported by Pouradier & Venet (1952) from observations on intrinsic-viscosity changes. These workers consider, in particular, that by the time the molecular weight becomes about 7000 the remaining bonds are more resistant than those already broken, but there is no evidence of such a limit from N-terminal residue data.

It can be seen from Table 3 that the N-terminal residues developed correspond in no instance to exhaustion of the residues available, but it is possible that, when account is taken of the C-terminal residues involved, particular bonds may be found to have been exhausted.

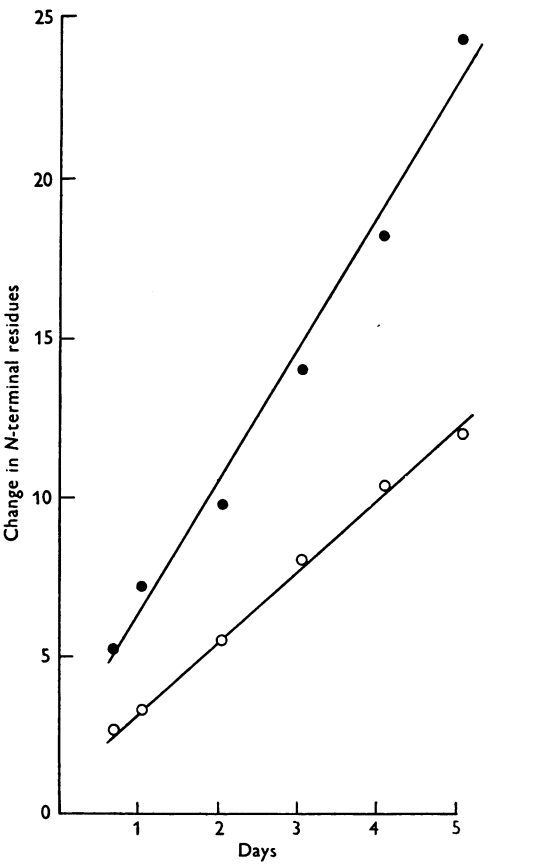

Fig. 4. Change in N-terminal residues with time after heating gelatin in solution at 90° and at constant pH. ●, pH 4·3; ○, pH 5·1. Residues as moles/1 000 000 g. gelatin.

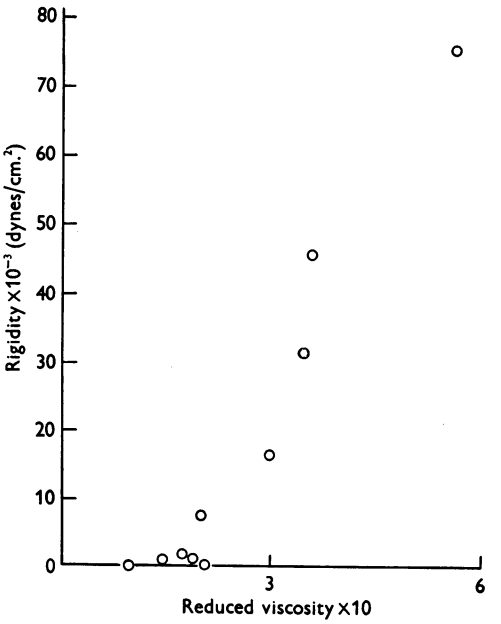

Fig. 5. The relationship between rigidity of gelatin gels and the reduced viscosity of the corresponding solutions.

The extent of degradation assessed by determination of N-terminal residues has been expressed as the percentage ratio $M_n/(M_n$ initial) (Tables 2, 4, 5) and compared with the results obtained by reduced viscosity, $\eta_t/(\eta_t$ initial). This is effectively a comparison between change in the number-average and weight-average molecular weights, and shows the type of relationship which might be expected.

If the results for rigidity are plotted against relative viscosity (Fig. 5) a consistent curve is not obtained. This is not surprising since it has already been established (Stainsby, Saunders & Ward, 1954) that the variation in rigidity is not solely related to changes in molecular weight.

SUMMARY

1. The N-terminal residues formed during degradation of gelatin under various conditions have been studied.

2. It is shown that the relative lability of different types of peptide bond varies with the pH of degradation.

3. The mean activation energy for peptide-bond hydrolysis at pH 5·1 is calculated as 18 960 cal./mole.

4. The extent of degradation, as measured by N-terminal residues, is compared with values obtained viscometrically. This is effectively a comparison between changes in number-average and weight-average molecular weights.

5. The kinetics of peptide-bond hydrolysis are discussed in relation to certain previous work.

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